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Structural Analysis and Identification of Novel Isoforms of the Circadian Clock Gene *period* in the Silk Moth *Bombyx mori*

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ABSTRACT—The molecular basis of the circadian clock is an autoregulatory feedback loop in which the PAS domain-containing protein PERIOD periodically inhibits its own transcription. In the present study on PERIOD of the silk moth *Bombyx mori*, we have cloned two distinct *period* mRNA homologues with different PAS domain sequences either with or without the pentapeptide GTQEK. A *period* cDNA fragment first amplified by PCR exhibited a 15 bp-deleted nucleotide sequence in the PAS domain, compared with the database sequence. A possible alternative splicing mechanism was examined by PCR analyses, and a 15 bp-inserted clone was also amplified. The entire sequences of these *period* α and *period* β isoforms were then determined by the 3’ and 5’ RACE methods. Isoform *period* α consists of a 3,324 bp oligonucleotide encoding 1,108 amino acid residues, whereas isoform *period* β comprises 3,309 bp corresponding to 1,103 amino acids. Isoforms *period* α and *period* β were found to be exactly identical except for the 15 bp deletion/insertion site. Such a pair of isoforms with a deletion/insertion sequence, namely two splice variants, has previously been reported only for the PERIOD proteins of the two honeybees, *Apis mellifera* and *A. cerana*. The occurrence of an alternative splicing mechanism in the *B. mori* *period* gene was hypothesized based on the genome structure recently clarified. *Bombyx mori* PERIOD α and β proteins are the isomers that reveal firstly the different PAS domain sequences.

Key words: circadian rhythm, cDNA cloning, alternative splicing, isoforms

INTRODUCTION

Circadian clocks are endogenous timing mechanisms that generate daily rhythms in diverse organisms from cyanobacteria to humans. Although rhythms controlled by circadian clocks are self-sustained and persist robustly with a period close to 24 hours under conditions of constant darkness and temperature, they are entrained by environmental time cues (zeitgebers) such as light, temperature, and food intake (Soriano, 1981; Rusak et al., 1993). The *period* gene has been demonstrated to play a crucially important role in circadian rhythms of both eclosion behavior and locomotor activity in the fruit fly *Drosophila melanogaster* (Konopka and Benzer 1971), the giant silk moth *Antheraea pernyi* (Sauman et al., 1996), and the silk moth *Bombyx mori* (Itoh et al., 1995). The major role played by *period* genes has been clarified by analysis of various types of mutants.

Circadian oscillations of a wide variety of organisms from insects to vertebrates are controlled through autoregulatory feedback loops involving PERIOD gene expression (Dunlap, 1999). In *Drosophila*, the lateral neurons (LNs), candidates for the fly’s circadian pacemaker, in addition to *period* also express a series of other clock genes, such as *timeless, clock, and cycle* (Shirasu et al., 2003). Each corresponding protein except for TIMELESS produced from *timeless*, namely, PERIOD, CLOCK, and CYCLE, respectively, contains a so-called PAS domain that enables the molecule to construct rigid heterodimers. The PERIOD/TIMELESS dimer is translocated from the cytoplasm to the nucleus to function as a negative regulator of its own positive transcription factor CLOCK/CYCLE (Saez and Young, 1996; Lee et al., 1999).

The importance of the PAS domain for dimerization has...
been shown by mutations at the PERL site and PAS B repeat regions, both of which substantially decreased dimerization efficiency (Huang et al., 1993; Gekakis et al., 1995).

The PAS domain is an important signaling module not only for protein-protein interaction but also for monitoring several environmental changes such as light, oxygen, and redox potential (Getzoff, 2002). The PAS domain itself would be exposed in the isolated PERIOD protein, but should be covered by the binding partner protein when PERIOD is in a dimer configuration.

In addition to dimerization with TIMELESS, the PERIOD protein may also interact with CLOCK or CYCLE to suppress the transcriptional function of the CLOCK/CYCLE dimer. PERIOD may also exist as a homodimer, though no physiological evidence has yet been reported on its function in that configuration (Huang et al., 1993). It is thus likely that PERIOD possesses a molecular contrivance to select a counterpart protein. In our effort to identify the clock genes and their proteins in the silk moth Bombyx mori, we initiated the cDNA cloning of the clock protein PERIOD. When the region corresponding to the PAS domain was amplified by PCR, a 15 bp-deleted nucleotide sequence was identified with respect to the sequence reported in the GenBank database (accession number AF063429). This 15 bp nucleotide sequence corresponds to the amino acid sequence GTQEK. PAS domain sequences are highly conserved among the various insect PERIOD homologues (Regier et al., 1998).

When we aligned the sequence of PAS domains very carefully, however, the domains were classified into two groups: namely, PAS domains with GTQEK or a related pentapeptide and PAS domains lacking such a fragment (Fig. 1). All these results strongly suggested the occurrence of an alternative splicing mechanism in the B. mori period gene. Thus, in the present study, we carried out a full sequence analysis of possible alternative splicing mRNA products to confirm the presence of PERIOD isoforms that possess PAS domain with either a deletion or an insertion of a GTQEK pentapeptide. Here we report the cDNA cloning of two splice variants of B. mori PERIOD protein and their circadian profile of accumulation.

### MATERIALS AND METHODS

#### Animals

Silk moths (female) were obtained from Kyoya (Kyoto), and maintained in a light/dark LD 17:7 photoperiod at 25°C. To collect samples for cDNA cloning, B. mori brains and optic lobes were excised at ZT17 and ZT9 (ZT: Zeitgeber time denotes the time entrained by environmental time cues, whereby lights on is ZT0 and

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**Fig. 1.** Comparison of the primary structures of Lepidoptera PERIOD proteins aligned around the GTQEK-like sequence. The amino acid sequences in a black box represent GTQEK-like sequences. ‘*’ Residues in that column are identical in all sequences in the alignment. ‘.’ Semi-conserved substitutions have been observed.
lights off is ZT17). The samples were frozen in liquid nitrogen and stored at −80°C until use.

cDNA cloning with degenerate PCR
mRNA was purified using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences; Piscataway, NJ, USA) according to the manufacturer’s instructions. For the extraction of mRNA, frozen brains and optic lobes described above were homogenized in a buffer solution containing guanidinium thiocyanate. The extract was diluted by 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, then the solution was incubated with oligo deoxythymidylic acid dT17-attached cellulose to bind the poly A of mRNA molecules. After the cellulose was washed, extracted mRNA was reverse transcribed into cDNA by dT17-adapter primer (5'-GGCCACGCAGTC-GA CTAGTAC-dT17-3') using AMV reverse transcriptase (Promega, Madison, WI, USA) as previously described (Frohman et al., 1988). After phenol extraction followed by ethanol precipitation, the resulting single strand cDNA was used as a template for a subsequent PCR. The PCR mixture (100 µl) included 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 µM of each primer, 0.2 mM dNTP mix, and 2.5 units of DNA polymerase.

PCR was carried out by using a sense primer PAS-AF (5’-GGGWMGTCTVTTYAGAYTTTYGKCA-3’) and an antisense primer PAS-CR (5’-TCRTGRTGNGGHARDYHCDCVAGYAT-3’). These primers were designed with reference to highly conserved amino acid sequences of insect PERIODs aligned for the so-called C2 region (Colot et al., 1988). In these primers, the letters D, H, K, M, N, R, V, Y denote the nucleotides (not C), (not G), (G or T), (A or C), (any), (A or G), (not T), and (C or T), respectively. The reaction was performed on a Geneamp system 2400 (Perkin Elmer, MA, USA) using PLATINUM DNA polymerase (Invtrogen, Carlsbad, CA, USA) with the following conditions: 3 min at 94°C followed by 35 cycles denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 90 sec, and final extension for 10 min at 72°C. Single-primer control PCRs were also carried out in parallel. Two primer-specific PCR products were electrophoresed on a 1% agarose gel, and recovered by phenol extraction followed by ethanol precipitation. The gel-purified PCR products were subcloned into pCR 2.1-TOPO cloning vector (Invtrogen).

3’ RACE for identification of the 3’ end of Bombyx mori period mRNA
To amplify the 3’ end of B. mori period cDNA, we carried out 3’ RACE as described by Frohman et al. (1988) by using the primers designed from the identified fragment sequence of B. mori period cDNA. Reverse-transcribed cDNAs obtained above were amplified with a series of sense primers and an antisense adapter primer (5’-GGCCACGCAGTC-GACTAGTAC-3’) and antisense primer PAS-CR (5’-TCRTGRTGNGGHARDYHCDCVAGYAT-3’). These primers were designed with reference to highly conserved amino acid sequences of insect PERIODs aligned for the so-called C2 region (Colot et al., 1988). In these primers, the letters D, H, K, M, N, R, V, Y denote the nucleotides (not C), (not G), (G or T), (A or C), (any), (A or G), (not T), and (C or T), respectively. The reaction was performed on a Geneamp system 2400 (Perkin Elmer, MA, USA) using PLATINUM DNA polymerase (Invtrogen, Carlsbad, CA, USA) with the following conditions: 3 min at 94°C followed by 35 cycles denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 90 sec, and final extension for 10 min at 72°C. Single-primer control PCRs were also carried out in parallel. Two primer-specific PCR products were electrophoresed on a 1% agarose gel, and recovered by phenol extraction followed by ethanol precipitation. The gel-purified PCR products were subcloned into pCR 2.1-TOPO cloning vector (Invtrogen).

5’ RACE for identification of the 5’ end of Bombyx mori period mRNA
For amplification of the 5’ end of B. mori period cDNA, we performed 5’ RACE using rapid amplification of cDNA ends (version 2.0: Invitrogen) mostly according to the manufacturer’s protocol. First-strand cDNA was synthesized from mRNA isolated by using a gene specific primer bmperantisense1 (5’-GGAGTTAGTAGA-CATTTCCTCTTT-3’) (Fig. 1) and ThermoScript™ RNase H Reverse Transcriptase (Invitrogen) at 60°C. The product was digested by RNase H to remove the original mRNA template. The remaining dNTP, primer, and contained proteins were separated from synthesized cDNA by using a GLASSMAX® DNA Isolation Spin Cartridge System (GIBCO BRL®; Rockville, MD, USA). A homopolymeric tail poly(C) was attached to the 5’ end of purified cDNA by using terminal deoxynucleotidyl transferase and a substate dCTP in a PCR-compatible buffer.

PCR amplification was accomplished using PLATINUM Taq DNA polymerase (Invitrogen) and gene specific antisense primers such as bmperantisense2 (5’-TGCTCAGACTCTGTAGAC-3’), bmperantisense3 (5’-CCGACACACACATGTCATGCAA-3’) (Fig. 1) and deoxyinosine-containing primer primers were provided in the kit (GIBCO BRL®). PCR products with bmperantisense1 and Abridged Anchor Primer (AAP) (5’-GGCCACGCAGTC-GACTAGTAC-3’) was further amplified with bmperantisense2 and Abridged Universal Anchor Primer (AUP) (5’-GGCCACGCAGTC-GACTAGTAC-3’). These nested PCR amplifications were carried out under the following conditions: 3 min at 93°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec. PCR products were then electrophoresed on an agarose gel and the resulting pure product was subcloned into pDrive cloning vector.

PCR amplifications for identifying Bombyx mori period isoforms
To identify spliced and non-spliced clones, two PCR strategies were systematically performed, focusing on the expected sequence differences between the isoforms. The two predicted gene isoforms were denoted as period α including a 15 bp oligonucleotide sequence that corresponds to the pentapeptide GTEQK, and period β which lacks this sequence (Fig. 2). In the first PCR, a set of synthetic oligonucleotides of bmperGTQEK primer and bmperantisense1 primer were used to amplify period α specifically. In the second PCR, a set of bmperantisense3 (5’-GACCGTGACGT-TCGGCTCACAG-3’) and bmperantisense3 primers was used to amplify period β and period α simultaneously. These PCR products were carried out by PLATINUM® Taq DNA polymerase as follows: 3 min at 94°C followed by 35 cycles (29 cycles for the second PCR) of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. These PCR products were used for electrophoresis on a 2% agarose gel, and the gel-purified PCR products were subcloned into pBluescript II SK+ cloning vector (Stratagene) for subsequent sequence analyses.

Quantitative analyses by RNase protection assay and RT-PCR for Bombyx mori period mRNA
Bombyx mori brains and optic lobes (10 each) were collected every 4 h for a full day. Total RNAs were extracted by centrifugation using a silica column. RNase protection analyses was performed using 5 µg of total RNA from each time-point. A cDNA of ribosomal protein 49 (abbreviated here as: RP49 for the protein, and rp49 for the gene) was utilized as an internal standard for mRNA transcription. [32P]UTP-labeled rp49 complementary RNA antisense probe was prepared by the in vitro transcription driven from the T3 RNA polymerase promoter. The period cDNA fragment obtained by PCR amplification using the primer bmperantisense2 and antisense primer...
(5'-AAGCATTACCGAATCACGC-3') was digested by restriction enzyme EcoRI and then the smaller fragment was subcloned into pBluescript II SK+ cloning vector (Stratagene). The plasmid was linearized by restriction enzyme XhoI and [32P]UTP-labeled period complementary RNA antisense probe was prepared by the in vitro transcription driven from T3 RNA polymerase promoter. The period complementary RNA antisense probe was prepared by the in vitro transcription driven from T3 RNA polymerase promoter.
riboprobe protected nucleotides 1526–1844 of the period cDNA. The sample of total RNAs was resuspended in a hybridization buffer (40 mM piperazine-1,4'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 400 mM NaCl, 1 mM EDTA in the final solution) containing a set of probes (50,000 cpm each of rp49). Hybridization was carried out at 45°C overnight after heat denaturation at 85°C for 3 min. The reaction mixture was treated with RNases A and T1 at 13°C for 30 min, and then the digest was treated with phenol/chloroform followed by centrifugation. The supernatant was treated with EtOH to precipitate RNA. Protected double-stranded RNAs were separated on a gel of 6% acrylamide with 8 M urea and visualized by autoradiography. Quantification of the protected fragments was performed with a Lane and Spot Analyzer (ATTO, Tokyo) by calibrating the amount of period relative to that of rp49. Two independent experiments were performed with similar results.

A semi-quantitative RT-PCR was carried out by amplifying the actin gene as an internal standard. Primers used were a gene-specific sense primer bmactin1 (5'-AGTGCAAGGCGGTCTTGC-3') and an antisense primer bmactin2 (5'-GCAGCAAGGCTCATTTGTAG-3'), which correspond to nucleotides 49–68 and 332–341 respectively (Nicole et al., 1987). The actin gene was amplified under the following conditions: 3 min at 94°C, followed by 28 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. PCR for the period gene was performed with bpmersense3 and bmperantisense1 as follows: 3 min at 94°C followed by 22 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. These PCR products were analyzed by electrophoresis on a 2% agarose gel.

Sequence analyses
Sequence analysis was carried out on a Thermocycler Cy5.5 dye terminator cycle sequencing kit (Amersham Biosciences) to determine the nucleotide sequence of plasmid DNA containing period. All the subcloned PCR products described above were analyzed from both 5' and 3' directions. For sequence determination, at least three individual clones were analyzed.

RESULTS
Isolation of a Bombyx mori period cDNA fragment
The first PCR amplification with a pair of the primers PAS-AF and PAS-CR for Bombyx mori cDNA afforded an oligonucleotide fragment with 969 bp (Fig. 2). The deduced sequence with 323 amino acids was judged to be the region corresponding to PAS domain from its similarity to those of other insect PERIOD PAS domains. When this amino acid sequence was compared with the sequence reported by Regier et al. (1998), however, we found that the sequence of Bombyx mori PERIOD reported in the database contained an additional five amino acid residues in this PAS domain. The insertion/deletion of GTQEK pentapeptide was found between the Asn(=N) and Ala(=A) residues in a proline-truncated site PKKNAQSP.

As shown in Fig. 1, there is a group of PERIOD proteins with GTQEK or related peptides in the PAS domain, while there is another group of PERIOD proteins lacking GTQEK at the same site. This strongly suggested the presence of an alternative splicing mechanism for the insertion/deletion of the pentapeptide. When the database oligonucleotide sequence was examined carefully, it was indeed found that the B. mori period mRNA consisted of consensus splicing sequences at this particular site (see Discussion). To confirm the existence of PERIOD with a proline-truncated site PKKNAQSP, we further carried out PCR experiments to identify a clone containing the oligonucleotide fragment corresponding to the pentapeptide inserted.

Identification of novel period isoforms that differed in their PAS domain open reading frame
In order to amplify a clone consisting of oligonucleotide GTACGCAAGAAAAG that encodes GTQEK, we carried out PCR by using a set of primers of bmperGTQEK and bmperantisense1 (Fig. 2). The primer bmperGTQEK that contains an oligonucleotide fragment GTACGCAAGAAAG (=GTQEK) is expected to hybridize to a clone of cDNA isoform that is being explored. As a result, a single cDNA of approximately 193 bp was amplified (Fig. 3A), and the sequence analysis of this PCR product revealed indeed the presence of 15 bp oligonucleotide corresponding to the pentapeptide GTQEK. The result clearly indicated that a clone having GTQEK or related peptides in the PAS domain, while there is another group of PERIOD proteins lacking GTQEK at the same site. This strongly suggested the presence of an alternative splicing mechanism for the insertion/deletion of the pentapeptide. When the database oligonucleotide sequence was examined carefully, it was indeed found that the B. mori period mRNA consisted of consensus splicing sequences at this particular site (see Discussion). To confirm the existence of PERIOD with a proline-truncated site PKKNAQSP, we further carried out PCR experiments to identify a clone containing the oligonucleotide fragment corresponding to the pentapeptide inserted.

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tinct protein isoform in the silk moth B. mori.

When PCR was carried out with bmperantisense3 and bmperantisense3 at ZT 17. Black arrowheads indicate period α, while white arrowhead shows period β.

**Fig. 3.** Electrophoresis profiles of PCR amplification products for detection of the alternative splicing site. (A) Lane 1: marker; and Lane 2: Amplification product of period α specific band corresponding to 193 bp with bmperGTQEK primer and bmperantisense1 primer. (B) Lane 1: marker; Lane 2: Amplification of the gel bands corresponding to 102 bp period α and 117 bp period β oligonucleotide fragments, respectively, with bmpersense3 and bmperantisense3 at ZT 17. Black arrowheads indicate period α, while white arrowhead shows period β.

In order to confirm the amplification of a unique period α fragment, another nested PCR was carried out with AUAP and a period α-specific primer bmperantiGTQEK. The primer bmperantiGTQEK has an antisense sequence against an oligonucleotide corresponding to the entire GTQEK, and was expected to hybridize to the clone of the period α cDNA. Eventually, the sequence of period α 5’ region was ascertained, by repeatedly analyzing a number of clones. The overlapped oligonucleotide sequences of period α and period β isoforms were found to be exactly identical. In addition to the 15 bp corresponding to GTQEK, a sequence difference between period α and period β was seen for the 5’ end in the 5’ UTR. The isomer period α possesses about a 65 bp longer UTR at the 5’ terminal (Fig. 2).

3’ RACE to determine the 3’ end sequence of Bombyx mori PERIOD cDNA isoforms

To obtain individual period α and period β cDNA clones including 3’ UTR, 3’ RACE by the touchdown PCR procedure was first performed using a gene-specific sense primer bmpersense1 and adapter primer. Subsequent nested PCR was carried out with another sense primer bmpersense2 and adapter primer. These sense primers bmpersense1 and bmpersense2 were designed for the region upstream of the alternative splice site, and thus amplified PCR products should comprise both oligonucleotide fragments from period α and period β (Fig. 2). PCR products isolated from a gel band of approximately 2,800 bp in electrophoresis were subcloned into the cloning vector for sequence analysis. When several clones were analyzed randomly, exactly the same nucleotide sequence was clarified from a set of clones and it was judged to be a complete 3’ terminal fragment of period β. This fragment lacked the 15 bp that corresponds to GTQEK. On the other hand, another set of clones was also sequenced and identified to be a 3’ terminal fragment of period α. These included a 15 bp oligonucleotide sequence. Collectively, the sequences analyzed by the 3’ RACE method for fragments of period α period β were found to be exactly identical except for the alternative splicing site of 15 bp.

The entire sequences of these period α and period β isoforms were thus determined by the 3’ and 5’ RACE methods. In summary, isoform period α consists of a 3,324 bp oligonucleotide encoding 1,108 amino acid residues, whereas isoform period β comprises 3,309 bp corresponding to 1,103 amino acid residues. Isoforms period α and period β were found to be indistinguishable with the exception of the 15 bp deletion/insertion site. Such a pair of isoforms with a deletion/insertion sequence, namely two splice variants, has pre-
Isoforms of Clock Protein PERIOD 909

viously been reported only for the PERIOD proteins of the two honeybees, Apis mellifera and Apis cerana japonica (Toma et al., 2000; Shimizu et al., 2001).

In these 3’ RACE reactions, a band of smaller molecular size was often observed in the gel electrophoresis. If we were to carry out nested PCR using sense primer bmpersense4 designed downstream from the alternative splice site, amplified PCR products should comprise only a single oligonucleotide fragment from both period α and period β. Our careful search for the cDNA sequence found a fragment (5’-GTCCTAGTCGACGCGTGCCG-3’) near the stop codon, to which the antisense nucleotide sequence of adapter primer was very much similar. In particular, 5’ terminal sequences of 11 nucleotide residues were almost completely identical. When an adapter primer was utilized as an antisense primer, one major amplified product was obtained with a small amount of sub-product having about 200 bp less than the major product. When we utilized a dT17-adapter primer, no such sub-product was detected. These results clearly show that mis-hybridization occurred with the solo adapter primer, while the addition of dT17 prevented this unnecessary hybridization.

RNase protection assay for exploration of circadian oscillation of period mRNA

The fruit fly Drosophila melanogaster and the silk moth Antheraea pernyi both exhibit a prominent circadian oscillation of period mRNA, which functions for example for circadian rhythmicity in eclosion behavior and locomotor activity. In order to explore such a temporal pattern of mRNA abundance of the period gene in B. mori brains and optic lobes, the RNase protection assay was performed. Since the assay to differentiate gene isoforms appeared to complicate the outcome, we set a probe at a site away from the dele-

![Fig. 4. Cycling of period mRNA levels in Bombyx mori. RNA protection assay for period mRNA expression was carried out twice independently. The white-black bar represents the timing of the 17L:7D light-dark cycle experienced prior to eclosion. Relative level (%) of mRNA refers to the ratio of period : rp49 mRNAs that were converted to percentage of maximal level.](image)

tion/insertion site. The radio-labeled B. mori rp49 and period cRNA antisense probes were hybridized with the respective mRNA obtained at 4-h intervals throughout the 24-h cycle and then the protected fragments were electrophoresed. The autoradiograph intensities of the gel bands were assessed by calibrating the amount of period relative to that of rp49 (Fig. 4). RP49 served as a control for the amount of RNA loaded in each lane. It was found that the B. mori period mRNA also fluctuated in a circadian manner with the highest mRNA level in the early night (around ZT 17) and the lowest level in the day (around ZT 13), showing a cycle similar to those seen in the period mRNA expression of other insects. The magnitude of the daily mRNA oscillation was about 3.5-fold.

**DISCUSSION**

**Sequence similarity and identity of PERIOD proteins**

Whether or not the structurally elucidated Bombyx mori proteins are PERIOD was examined by comparing their whole structures, aligning the sequences with other reported PERIOD proteins. When the full-length sequences were compared, B. mori PERIOD showed considerably high homologies with other insect PERIOD proteins. Calculated sequence similarity and identity are 48% and 29% for Drosophila melanogaster PERIOD, 56% and 42% for Antheraea pernyi PERIOD, and 73% and 58% for Danausplexippus PERIOD. PERIOD proteins are distinguished by having a series of characteristic conserved (C) domains numbered C1–C7, and C1–C3 are particularly highly homolo-

gous among insect PERIOD proteins (Colot et al., 1988; Lin et al., 2002). As shown in Fig. 5, these C1–C3 characteristic regions are highly conserved in the B. mori PERIOD proteins clarified in the present study. It should be noted that B. mori PERIOD proteins are significantly highly homolo-

gous with Danausplexippus PERIOD in their entire struc-

tures.

PERIOD proteins also have several other signals and domains that are functionally important in exhibiting potential cycling activities. When the primary structure of B. mori PERIOD proteins was depicted by a rod model as shown in Fig. 6, a domain construction was found to be almost the same as other insect PERIOD proteins. These include the nuclear localization signal (NLS) (Vosshall et al., 1994; Chang et al., 2003; Chang and Reppert, 2003), the PAS domain (Crews et al., 1988), the cytoplasmic localization domain (CLD) (Saez and Young, 1996), and the nuclear export signal (NES) (Fukuda et al., 1997; Vielhaber et al., 2001). It was found that all of these sequences are highly conserved in B. mori PERIOD proteins with 70–100% sequence similarities. In particular, the most important characteristic domain structure PAS showed considerably high homologies: i.e., calculated sequence similarity and identity for PAS-A and PAS-B domains, (88% similarity and 69% identity for PAS-A) and (67% similarity and 38% identity for
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C1

D. m.  YK5c
M. d.  KX5c
P. a.  N5c
B. g.  S5c
A. m.  E5c
A. p.  T5c
D. p.  U5c
B. m.  V5c

PAS-A

D. m.  YK5c
M. d.  KX5c
P. a.  N5c
B. g.  S5c
A. m.  E5c
A. p.  T5c
D. p.  U5c
B. m.  V5c

C2

PAS-B

D. m.  YK5c
M. d.  KX5c
P. a.  N5c
B. g.  S5c
A. m.  E5c
A. p.  T5c
D. p.  U5c
B. m.  V5c

CLD

NES

D. m.  YK5c
M. d.  KX5c
P. a.  N5c
B. g.  S5c
A. m.  E5c
A. p.  T5c
D. p.  U5c
B. m.  V5c

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Fig. 5. PERIOD protein sequences alignment comparison among eight insect species. PERIOD protein sequences are aligned by inserting gap (−) to achieve maximum homology. Functional motif structures such as NLS, PAS-A, PAS-B, NES, and CLD are boxed. The defined C1–C7 regions are underlined with labels below. Seven different insect PERIOD protein homologues are aligned with *Bombyx mori* (**B.m.**), *Drosophila melanogaster* (**D.m.**), *Musca domestica* (**M.d.**), *Periplaneta americana* (**P.a.**), *Blattella germanica* (**B.g.**), *Apis mellifera* (**A.m.**), *Antheraea pernyi* (**A.p.**), and *Danaus plexippus* (**D.p.**). '*'Residues in that column are identical in all sequences in the alignment. ':'Conserved substitutions have been observed. '.'Semi-conserved substitutions have been observed.
PAS-B) for *Drosophila melanogaster* PERIOD, (95% and 83% for PAS-A) and (88% and 71% for PAS-B) for *Antheraea pernyi* PERIOD, and (96% and 86% for PAS-A) and (85% and 75% for PAS-B) for *Danaus plexippus* PERIOD. The results clearly indicate that the proteins elucidated in this study are genuine PERIOD proteins.

In the present study, we were able to clone two different *B. mori* period cDNAs. The structures of their open reading frames were completely elucidated and these isoforms showed totally identical sequences except for a deletion/insertion difference of 15 bp. Thus, our initial purpose to find a clone having a fragment corresponding to the pentapeptide GTQEK was successfully achieved by cloning an isoform containing this 15 bp. The resulting PERIOD α and β proteins are the first period transcript pairs with different PAS domain sequences present in a single insect species.

A similar set of PERIOD isoforms has been reported only for the honeybees, *Apis mellifera* and *Apis cerana*, but in that case for a deletion/insertion of eight amino acid residues near the so-called *per* mutation site (Shimizu *et al*., 2001).

Most recently, genome structural analyses have been reported for the silkworm *Bombyx mori* (Mita *et al*., 2004) and the genome sequence has been opened as an Internet web site (http://sgp.dna.affrc.go.jp). Even though sequence data are said to include only up to about 80% of protein encoding genes, we attempted a BLAST search of genomic DNA sequences using our cDNA sequence in order to reveal the genomic period. We hit a series of fragments, but not a single gene, that cover the entire period cDNA sequence elucidated in this study. We found three cDNA regions that are absent from the genome DNA sequences, i.e. two regions in the open reading frame with about 42 bp and 90 bp corresponding to 14 and 30 amino acid residues, respectively, and one region in 5' UTR with about 48 bp. It is thus evident for period that although the genome sequence is incomplete at the moment, the cDNA structure we have clarified is almost completely compatible with the genome sequence and that of 3,800 bp-overlapped regions, only 9 mismatched nucleotides were found.

**Alternative splicing mechanism to afford α and β PERIOD isoforms**

In the present study, we identified novel period isoforms that possess a deletion/insertion of 15 bp in the PAS domain. The cDNA structure of period α clearly exhibited the existence of splice consensus sites (Norton *et al*., 1994; Maniatis and Tasic, 2002), i.e. GU (GT in cDNA) at the 5' side and AG at the 3' side of the specific 15 bp sequence GTACGCAAAGAG. This strongly suggests that the isoforms with the 15 bp sequence difference were produced by either ordinary processing (α) or by alternative splicing (β). Alternative splicing would be brought about from an intron longer than 15 bp or from DNA itself. Since the genome sequence indicates that there is an intron adjacent to this splicing site, the alternative splicing mechanism illustrated in Fig. 7 is speculated as shown. It is clear that GT and AG splicing consensus sites truncate the intron. At this moment, the structural trigger to cause this mechanism is not known.

As shown in Fig. 1, PERIOD proteins having GTQEK-like sequences have been found in a series of species of Lepidoptera. In particular, PERIOD proteins of *Apatelodes*, *Danaus*, and *Plodia*, consist of exactly same pentapeptide GTQEK. However, no isoform lacking GTQEK has so far been reported for any of the PERIOD proteins having a GTQEK(-like) sequence, and it is not clear at this moment whether their isogene is simply missing. When we carefully examined the oligonucleotide sequences corresponding to these GTQEK(-like) sequences, however, it was found that the splice consensus sites existed in all genes (Fig. 8). Some Lepidopteran period genes clearly show a distinct 15 bp GT–AG fragment, while some other genes exhibit more than 15 bp of GT–AG fragments. Of course, this so-called consensus frame sequence for splicing is not always essential for alternative splicing mechanisms. Moreover, PERIOD proteins devoid of GTQEK(-like) sequences have also been isolated from several Lepidoptera insects (Fig. 1). There is a possibility that these PERIOD proteins are the products of alternative splicing and thus there are isoforms containing GTQEK(-like) sequences. No reports have been announced for such splice variants, however.

When the secondary-structure of the PAS domain of *B. mori* PERIOD isoform α was analyzed by the Chou-Fasman
method (Nishikawa and Noguchi, 1991; Ito et al., 1997), GTQEK in a proline-truncated tetradecapeptide PKTANGTQEKAQSP frame was predicted likely to be in a loop structure. The particular PAS site of B. mori PERIOD isoform β, PKTANAAQSP lacking GTQEK is also in a loop. These loop structures may be exposed to the molecular surface when the protein is not bound in a complex. The presence or absence of GTQEK might therefore affect the preferred interaction with other proteins, for example TIMELESS. The side chains of T(=Thr), Q(=Gln), E(=Glu), and K(=Lys) are all hydrophilic and act simultaneously as both proton donor and acceptor.

Fig. 7. The putative mechanism of alternative splicing to produce period α and period β mRNA, respectively, from premature Bombyx mori period mRNA. The letters in the parentheses, n and n + 1, denote the number of exon in the period gene.

Fig. 8. Nucleotide sequence and its deduced amino acid sequence of various Lepidoptera PERIOD proteins around GTQEK-like site. The nucleotide sequences depicted in Fig. 1 are analyzed in the putative alternative splicing site (gray letters). It is probable that a PERIOD isoform lacking GTQEK or GTQEK-like sequence is present in each insect.

Questions to be answered for α and β PERIOD isoforms
Although we may have identified two distinct mRNA, or protein isomers of PERIOD α and β, there are still a number of questions to be answered. The first question is whether or not the presence of these isoforms is a simple consequence of individual differences between silk moths. Since we found a splice site for pentapeptide GTQEK in the exon of the silkworm genome DNA sequence, it is likely that there
is a specific alternative splicing mechanism to produce either period α mRNA or period β mRNA. If such a mechanism is dependent upon the individual silk moth, there might be no significant functional difference between PERIOD α and β. In order to solve these questions, it will be necessary to quantify the ratio of isoform α and isoform β in PERIOD protein forms or period mRNA forms. So far, we have not succeeded in such quantifications in spite of attempts to differentiate the ratio between period α and period β mRNAs. As shown in Fig. 3B, the amount of period β mRNA was judged to be a few times larger than that of period α mRNA. To quantify this difference more firmly, we may need an elite system to amplify each mRNA isoform accurately and quantitatively.

The RNase protection assay performed for period mRNA exhibited a distinct temporal pattern of mRNA abundance of the B. mori period gene with an approximately 3.5-fold daily oscillation in the amount of mRNA. The question therefore arose as to whether the isomers exhibited the same temporal pattern of such oscillation. Using a semi-quantified PCR method, we attempted to quantify the period mRNA isoforms, but were unable to measure the amount of each isoform accurately, so that the ratio varied considerably, resulting in our inability to see a prominent oscillation. The period β mRNA is several times more abundant than period α mRNA throughout the entire day. Again, we need a selected system to amplify each mRNA isoform precisely and quantitatively.

Another important issue is whether these two period mRNA isoforms are expressed in the same neurons. We first attempted in situ hybridization for adult silk moth brains by using probes prepared from various sites of the cDNA clone. However, this ordinary hybridization is not yet successful for reasons that are not yet clear. On the other hand, isoforms of the PERIOD proteins should be detected by sophisticated monoclonal antibodies that can differentiate the insertion/deletion of pentapeptide GTQEK, so that the immunochemical study could then clarify their localization in different cells. Thus, we are now preparing antibodies for further investigation of the PERIOD protein. Nonetheless, the present study has shown, for the first time, the presence of two period transcripts with different PAS domain sequences present in a single insect species. It will now be important to analyze or specify the functions of the two isoforms, whether these may be different or differently localized, especially in order to explore the essential molecular mechanism of circadian rhythms in this species.

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